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- Schiff base complex shows that Wat<sup>21</sup> is equivalent to Wat<sup>29</sup>. Also in the native structure, a water molecule is found in the identical position (within 0.5 Å of Wat<sup>29</sup> of the carbinolamine). Wat<sup>72</sup> in the Schiff base complex could correspond to the water molecule derived from protonation of the hydroxyl group of the carbinolamine.
25. Known class I structures used for comparison were KDPG aldolase in complex with pyruvate [Protein Data Bank (PDB) code 1EUA] (17), human muscle fructose 1,6-bisphosphate aldolase (PDB code 4ALD) (19), rabbit muscle 1,6-bisphosphate fructose aldolase (PDB code 1ADO) (20), and transaldolase B (PDB code 1UCW) (21).
  26. This mutant crystallized under the same conditions as the wild type, and the substrate soak was repeated as reported in Table 1.
  27. This observation was also made for the rabbit aldolase A, where the Lys<sup>146</sup> → Arg<sup>146</sup> mutant retained the ability to form the Schiff base intermediate (43).
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  30. Experimental conditions for the pH activity profile were as follows: 25 mM buffer solutions of sodium formate (pH 3.5, 3.0, and 4.0); sodium acetate (pH 4.5, 5.0, and 5.5); MES (pH 5.5, 6.0, and 6.5); MOPS (pH 6.5, 7.0, and 7.5); tetraethylammonium-chloride (TEA-HCl) (pH 7.5, 8.0, and 8.5); CAPSO (pH 8.5, 9.0, and 9.5), and CAPS (pH 10, 10.5, and 11.0).  $V_{max}$  was measured from pH 4 to 10 in the retroaldol direction with 3 mM DRP in 50 mM (pH 7.5) TEA-HCl buffer in the presence of 0.3 mM reduced nicotinamide adenine dinucleotide (NADH) using a glyceraldehyde 3-phosphate dehydrogenase/triosephosphate isomerase (GPD/TP1)-coupled (5.3 U/ml, Sigma G-1881) enzyme system at 25°C by observing the rate of decrease of NADH concentration as monitored at 340 nm (44).
  31. In rabbit aldolase A, the equivalent Lys<sup>146</sup> residue has been implicated as being involved in cleavage and condensation of the C3–C4 bond of fructose 1,6-bisphosphate (45), in addition to lowering the  $pK_a$  of Lys<sup>229</sup>.
  32. All solvent-accessible surface areas were calculated with the program MS (46) with a 1.4 Å probe sphere and standard atomic radii (47).
  33. Experimental conditions for the Schiff base trapping experiment were as follows: DERA (1 mg/ml) was incubated with 5 mM acetaldehyde in 20 mM TEA-HCl, 50 mM NaCl, and 2 mM CaCl<sub>2</sub> (pH 7.4) at 22°C for 10 min. Fifty mM NaBH<sub>4</sub> was added, and incubation continued for 12 hours. Samples were dialyzed against dH<sub>2</sub>O and then purified by high-performance liquid chromatography on a C18 column before analysis by electrospray ionization mass spectrometry (with a Perkin Elmer API III Sciex triple quadrupole). Observed masses agreed within ±4 daltons to theoretical values.
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  36. The activity of the class I aldolase from halophilic archaeobacterium *Haloarcula vallismortis* was not affected by carboxypeptidase digestion (48).
  37. The model was constructed with Molecular Simulations Insight 2000. Setup was as described (49). The CVFF force field provided in the Discover module of InsightII was used.
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  39. Experimental conditions for deuteropropanal synthesis and the DERA exchange experiment were as follows: (R)-2-deuteropropanol was synthesized from (S)-(+)-1,2-propanediol as described in (50) with minor modification. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz): 3.62 (d,  $J$  = 6.97 Hz, 2H), 1.60 to 1.55 (m, 1H), 0.91 (d,  $J$  = 7.34 Hz, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz): 64.58, 24.79 (t,  $J$  = 20 Hz), 9.91. (R)-2-deuteropropanol:  $[\alpha]_D^{25} = +1.11^\circ$  (CDCl<sub>3</sub>,  $c$  = 0.18); lit. +0.06° (neat). (S)-2-deuteropropanol was prepared analogously:  $[\alpha]_D^{25} = -0.05^\circ$  (CDCl<sub>3</sub>,  $c$  = 1.8); lit. -0.06° (neat). In an NMR tube, (R)- or (S)-2-deuteropropanol was incubated at 0.4 mM in 100 mM TEA-HCl buffer made up with D<sub>2</sub>O together with 8 mM pyruvic acid, 1 mM NAD<sup>+</sup>, yeast alcohol dehydrogenase (0.25 mg/ml) (82.5 U), and L-lactate dehydrogenase (0.25 mg/ml) (214.5 U), pH in D<sub>2</sub>O (pD) = 7.1. After the oxidation of deuteropropanol to deuteropropanal was deemed substantially complete by the appearance of the aldehyde C3 resonance by <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz): 1.08 (d,  $J$  = 7.34 Hz, 3H), then 0.5 mg/ml (50 U) wild-type DERA was added. For (R)-2-deuteropropanal, this resonance collapses to a singlet 1.04 (s, 3H) after 3 hours, whereas for (S)-2-deuteropropanal it remains unchanged.
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  59. Experimental conditions for propanal exchange with DERA mutants were as follows: In an NMR tube, propanal was incubated at 200 mM in 100 mM TEA-HCl buffer made up with D<sub>2</sub>O (pD = 7.1) and DERA (2 mg/ml) was added (50 U for wild-type DERA). Samples were analyzed by <sup>1</sup>H NMR (D<sub>2</sub>O, 250 MHz). For wild-type DERA, the C3-aldehyde triplet resonance 1.03 (t,  $J$  = 7.01 Hz, 3H) collapses to a doublet 1.02 (d,  $J$  = 7.3 Hz, 3H).
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  62. We gratefully acknowledge helpful discussions with F. Huang, S. Fong, L. Lee, T. Tolbert, P. Sears, W. W. Cleland, G. M. Sheldrick, J. H. Naismith, and R. A. Lerner. Supported by NIH grants GM44154 (C.H.W.) and CA27489 (I.A.W.), a Natural Science and Engineering Research Council of Canada postdoctoral fellowship (G.D.), and a UNCF-Merck Science Initiative fellowship (M.M.). We thank the Stanford Synchrotron Radiation Laboratory staff of beamline 9-2, X. Dai, and S. E. Greasley for help with data collection and processing, and M. Elsliger for computational assistance. This is publication 14200-MB from the Scripps Research Institute. The coordinates have been deposited in the PDB with access codes 1JCL (wild-type DERA) and 1JCJ (K201L mutant of DERA) and are available immediately from aheline@scripps.edu.

19 June 2001; accepted 2 August 2001

## Carboxyl-Terminal Modulator Protein (CTMP), a Negative Regulator of PKB/Akt and v-Akt at the Plasma Membrane

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The PKB (protein kinase B, also called Akt) family of protein kinases plays a key role in insulin signaling, cellular survival, and transformation. PKB is activated by phosphorylation on residues threonine 308, by the protein kinase PDK1, and Serine 473, by a putative serine 473 kinase. Several protein binding partners for PKB have been identified. Here, we describe a protein partner for PKB $\alpha$  termed CTMP, or carboxyl-terminal modulator protein, that binds specifically to the carboxyl-terminal regulatory domain of PKB $\alpha$  at the plasma membrane. Binding of CTMP reduces the activity of PKB $\alpha$  by inhibiting phosphorylation on serine 473 and threonine 308. Moreover, CTMP expression reverses the phenotype of v-Akt-transformed cells examined under a number of criteria including cell morphology, growth rate, and in vivo tumorigenesis. These findings identify CTMP as a negative regulatory component of the pathway controlling PKB activity.

PKB is a major downstream target of receptor tyrosine kinases that signal via the phosphatidylinositol 3-kinase (PI 3-kinase). PKB mediates a wide variety of biological responses to insulin and insulin-like growth factor 1

(IGF-1) and other growth factors (1–2). Upon cell stimulation, the kinase is translocated to the plasma membrane, where it is phosphorylated on two amino acids, Thr<sup>308</sup> in the catalytic domain and Ser<sup>473</sup> in the COOH-

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al regulatory domain (3–8). To date, proteins have been shown to physically interact with PKB (9–11).

To identify new proteins that interact with a HeLa cell cDNA library was screened by two-hybrid analysis, with the kinase in plus the COOH-terminal regulatory domain of PKB $\alpha$  as bait. From  $1.5 \times 10^6$  primary transformants screened, seven identical clones specifically interacted with PKB $\alpha$ , as demonstrated by activation of the reporters for histidine auxotrophy and lacZ activity [Web fig. 1, D and B (12)], and, in a mammalian cell two-hybrid assay (13). This interaction was observed with constructs containing the COOH-terminal regulatory domain of PKB $\alpha$ , encompassing amino acids 411 to 480 [Web fig. 1, C and 2)]. Sequence analysis revealed the presence of an open reading frame encoding a protein of 240 amino acids with a predicted

molecular mass of 27 kD that we termed COOH-terminal modulator protein, or CTMP (Fig. 1A). The cDNA contains the unusual feature of an Alu cassette at its 3' end, a sequence usually found in intronic DNA. Database screening of mouse expressed sequence tags revealed a protein of 230 amino acids with a similar sequence (79% identity) that may be the mouse homolog of CTMP (Fig. 1A). The mRNA for human CTMP was detected predominantly in skeletal muscle, testis, uterus, brain, and kidney, with lower levels observed in heart, liver, and lung [Web fig. 2A (12)]. The presence of multiple bands after reverse transcription-polymerase chain reaction (RT-PCR) strongly suggested that the gene for CTMP undergoes alternate splicing in some tissues, generating multiple RNA transcripts [Web fig. 3, A and B (12)]. Endogenous CTMP protein was detected with an antibody (14) specific for human CTMP in extracts from HeLa and human embryonic kidney (HEK) 293 cells, with weaker expression found in COS-1 cells [Web fig. 2B (12)]. Weaker CTMP expression was seen in the human SJRH30 rhabdomyosarcoma cell line, and no signal was detected in the rat H9C2 myocardium cell line. These data were confirmed by blotting with a second antibody against CTMP (13). CTMP migrated with apparent molecular masses of 22 to 26 kD in the different cell lines, possibly owing to post-

translational modification (see below).

When HeLa cell extracts were subjected to centrifugation into cytosolic (S100) and membrane (P100) fractions, endogenous CTMP was predominantly detected in the P100 fraction [Web fig. 2C (12)]. CTMP expression was detected in a range of different human cell lines (15), most notably in a glioblastoma cell line (LN229, Fig. 1C). Again, two molecular species corresponding to CTMP were observed in LN229 cells, and these forms appeared to be differentially localized to the membrane and cytosolic fractions of these cells (Fig. 1D). Immunofluorescence analysis demonstrated that endogenous PKB and CTMP colocalized at the plasma membrane (Fig. 1E). Endogenous complexes of CTMP and PKB were also detected in the P100 fraction of these cells by Western blotting (16). Immunolocalization of green fluorescent protein (GFP)-CTMP fusion protein indicated that CTMP associated with intracellular structures similar to membrane ruffles (Fig. 1B), whereas staining of the GFP control protein was detected in all cellular compartments. Time-lapse cine-microscopy of moving NIH 3T3 cells expressing GFP-CTMP revealed that the fusion protein was predominantly localized to the leading edge of the moving cells, decorating the rapidly moving membrane ruffles [Web fig. 4 (12)].

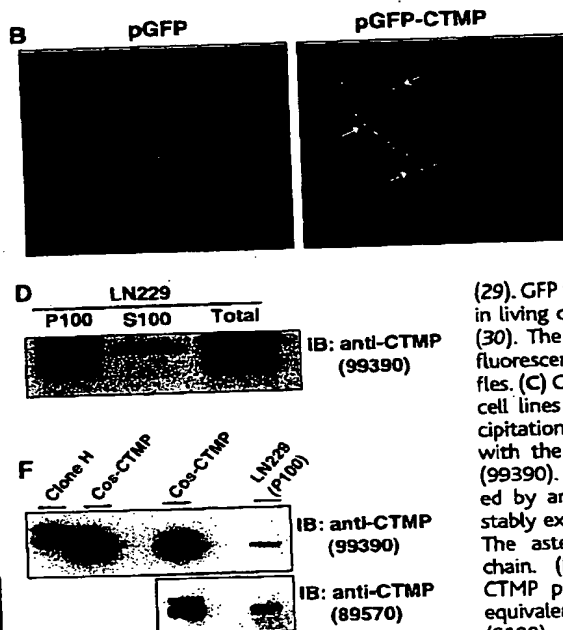
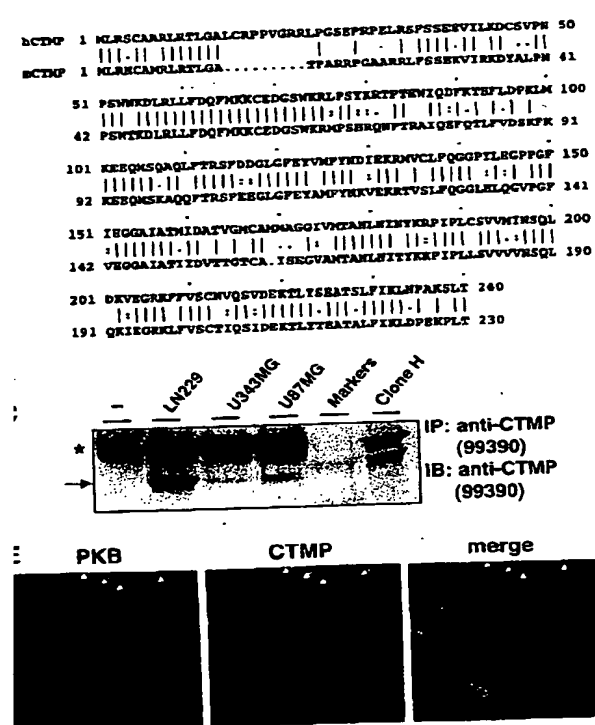
Western blotting of lysates from a control cell line stably expressing CTMP indicated that

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**Fig. 1. Structure and localization of human CTMP.** (A) Alignment of deduced amino acid sequences from human (accession number AJ313515) and mouse CTMP. (B) NIH 3T3 cells were transfected with 5 µg of expression vectors for GFP (pGFP, left panel) or GFP-CTMP (pGFP-CTMP, right panel)

(29). GFP fluorescence was then analyzed in living cells using confocal microscopy (30). The arrows indicate areas of high fluorescence intensity at membrane ruffles. (C) CTMP expression in glioblastoma cell lines was assessed by immunoprecipitation followed by immunoblotting with the same antibody against CTMP (99390). The position of CTMP is indicated by an arrow. Clone H is a cell-line stably expressing Flag-CTMP (see Fig. 5). The asterisk represents the IgG light chain. (D) Detection of endogenous CTMP protein by Western blotting of equivalent amounts (50 µg) of cytosolic (S100), membrane (P100) fractions (37)

or total cell lysate (Total) from LN229 cells. (E) To determine the cellular distribution of endogenous PKB $\alpha$  and CTMP proteins, LN229 cells were fixed with 4% paraformaldehyde and incubated with PKB mAb (32), followed by staining with protein A/G coupled to FITC (green, PKB panel), or with the polyclonal CTMP antibody 99390 followed by staining with

rhodamine-conjugated rabbit antibody (red, CTMP panel). Slides were analyzed by confocal microscopy, and the pictures represent the central section of the x-y plane. Areas of colocalization of CTMP and PKB at the membrane ruffles are visualized in yellow (merge panel) and are indicated by arrows. (F) CTMP from pHook2-CTMP transfected COS-1 cells (29), clone H cell extracts, or P100 fraction from LN229 cells was detected with CTMP antibodies 99390 and 89570 (14).







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## Isolation of the Protein Kinase TAO2 and Identification of Its Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase Binding Domain\*

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We previously reported the cloning of the thousand and one-amino acid protein kinase 1 (TAO1), a rat homolog of the *Saccharomyces cerevisiae* protein kinase sterile 20 protein. Here we report the complete sequence and properties of a related rat protein kinase TAO2. Like TAO1, recombinant TAO2 selectively activated mitogen-activated protein/extracellular signal-regulated kinase kinases (MEKs) 3, 4, and 6 of the stress-responsive mitogen-activated protein kinase pathways *in vitro* and copurified with MEK3 endogenous to Sf9 cells. To examine TAO2 interactions with MEKs, the MEK binding domain of TAO2 was localized to an ~135-residue sequence just C-terminal to the TAO2 catalytic domain. *In vitro* this MEK binding domain associated with MEKs 3 and 6 but not MEKs 1, 2, or 4. Using chimeric MEK proteins, we found that the MEK N terminus was sufficient for binding to TAO2. Catalytic activity of full-length TAO2 enhanced its binding to MEKs. However, neither the autophosphorylation of the MEK binding domain of TAO2 nor the activity of MEK itself was required for MEK binding. These results suggest that TAO proteins lie in stress-sensitive kinase cascades and define a mechanism by which these kinases may organize downstream targets.

clude mixed lineage kinases, TGF- $\beta$ -activated protein kinase, and TAO1 (3–8). In the yeast protein kinase family tree the Ste20p branch is closest to the MEK kinases (MEKKs) (9). Thus, it is not surprising that several mammalian Ste20p-related kinases are MEKKs. Some have selectivity for MEKs in the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway and others for the p38 stress-sensitive pathway, whereas most phosphorylate both groups of MEKs *in vitro* (3, 7, 8, 10–13). The plethora of Ste20p-like kinases with effects on stress pathways and their overlapping biochemical activities have made it difficult to define their roles in the physiological regulation of these kinase cascades. MEK3 and MEKK1 are almost certainly important for regulation of JNK/SAPKs because they bind to JNK/SAPK and other cascade components either through a scaffold protein, with a function believed to be analogous to the yeast scaffold protein Ste5p (14, 15), or directly (16, 17). The association of kinases in complexes provides compelling evidence for their interrelated or dependent functions even in the absence of information regarding physiological roles.

To identify novel components of MAP kinase cascades, we isolated several PCR products and cDNAs encoding homologs of Ste20p from *Saccharomyces pombe* and mammals (8, 18, 19). Among the mammalian cDNAs, we isolated one that encoded the protein kinase TAO1, named for its one thousand and one amino acids. TAO1 is like certain other relatives of Ste20p in that it phosphorylates and activates MEKs from the stress-responsive MAP kinase cascades. Copurification experiments indicated that TAO1 interacted with MEK3, a p38 activator, although direct binding was not demonstrated. These findings suggested that TAO1 forms complexes with components of p38 MAP kinase cascades and may, therefore, be an important regulator of p38-dependent events.

Here we report the isolation of cDNA clones encoding the complete sequence of TAO2, a close relative of TAO1. Both TAO1 and TAO2 are expressed most highly in brain cells, suggesting their tissue-restricted function (8). The *in vitro* substrate specificities of TAO1 and TAO2 are also similar. Importantly, TAO2, like TAO1, copurifies with MEK3 endogenous to Sf9 cells. This suggests that the intracellular specificity of TAO proteins may be determined by their ability to bind stably to a subset of potential MEK substrates. To define the mechanism by which TAO proteins associate with MEKs, we determined that they interacted directly, identified the MEK binding domain of TAO2, and examined the MEK specificity of this domain.

### EXPERIMENTAL PROCEDURES

**Isolation of cDNA Clones Encoding TAO2**—A 420-base pair PCR product was obtained as described (8) using oligonucleotides based on the yeast Ste20p sequence. This product was labeled with [ $\alpha$ - $^{32}$ P]dCTP by random priming and used to probe approximately  $1.3 \times 10^6$  plaques

Ste20p<sup>1</sup> was originally isolated as a gene of budding yeast whose product functioned downstream of the  $\beta\gamma$  subunits of a heterotrimeric G protein but upstream of enzymes in the MAP kinase module of the pheromone response pathway (1, 2). Several mammalian protein kinases related to Ste20p have been identified that phosphorylate MAP/ERK kinase (MEK) family members in stress-activated MAP kinase cascades. These in-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF140556.

† This work was submitted in partial fulfillment of the requirements for a doctorate of philosophy at the University of Texas Southwestern Medical Center.

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<sup>1</sup> The abbreviations used are: Ste20p, sterile 20 protein; MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; MEK, MAP/ERK kinase or MAP kinase kinase; MEKK, MEK kinase; TAO, thousand and one amino acid protein kinase; GST, glutathione S-transferase; MBP, myelin basic protein; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; PCR, polymerase chain reaction; DTT, dithiothreitol.

## MEK Binding Domain of TAO2

a random-primed adult rat forebrain cDNA library and approximately  $0.6 \times 10^6$  plaques of an oligo(dT)-primed rat brain cDNA library (provided by Jim Boulter, UCLA). cDNA clones encoding TAO1 and TAO2 were obtained. Subsequent rounds of screening yielded the full-length TAO2 cDNA, which was assembled into pBluescript from 3 or 5 positive clones. The complete sequence of the assembled cDNA was deposited in GenBank<sup>TM</sup> with the accession number F140556.

**Plasmid Construction**—pBluescript-TAO2-(1-320), containing the catalytic domain of TAO2, and a catalytically defective mutant pBlue-ript-TAO2D169A were generated by PCR. Wild-type TAO2, TAO2D169A, and TAO2-(1-320) were cloned into pRSETB (Invitrogen) to incorporate a MRGSH<sub>6</sub> tag and subsequently transferred into the baculoviral shuttle vector pVL1393. Recombinant viruses were selected as described (8). For expression in mammalian cells, the cDNAs encoding these TAO2 proteins were also cloned into pCMV5 that had been modified to place a Myc epitope tag at the N terminus of the encoded protein. A truncated, catalytically defective TAO2 in pRSETB was created by changing lysine 57, in the conserved VAIK motif, to alanine (K57A) by PCR.

For binding assays, fragments of TAO2 were subcloned into GEX-KG by PCR. TAO2-(314-451) was subsequently transferred into pRSETA utilizing the *Bam*HI and *Eco*RI restriction sites. Catalytically defective MEK3 was created in pNPT7-5 by changing lysine 64 to methionine (K64M). A MEK1/6 chimera, which contains the N-terminal domain of MEK1 and the C-terminal domain of MEK6, and a MEK6/1 chimera with the reciprocal domains (see Fig. 4B) were transferred into pRSETA or -C, respectively, from the original pGEX-KG-MEK1/6 and MEK6/1 plasmids (generously provided by Lori Christerson) utilizing the *Bam*HI and *Hind*III restriction sites.

**Expression and Purification of Recombinant Proteins from Sf9 Cells and Bacteria**—Recombinant histidine-tagged TAO2, TAO2-(1-320), and TAO2D169A were expressed and harvested from Sf9 cells as described previously for TAO1 (8). Proteins were adsorbed to Ni<sup>2+</sup>-nitrilotriacetic acid-agarose (Qiagen) and eluted with a gradient of 20–250 mM imidazole in 0.5 mM dithiothreitol (DTT) and 0.8 M NaCl. His<sub>6</sub>-TAO2D169A was further purified on MonoQ (Amersham Pharmacia Biotech) by elution with 50–450 mM NaCl in 1 mM DTT, 0.2 mM EGTA, 1 mM benzamidine, 10% glycerol, and 20 mM Tris, pH 8. TAO2 was detected by Western blotting with an antibody to the MRGSH<sub>6</sub> epitope (Qiagen) and silver staining. GST fusion proteins, His<sub>6</sub>-tagged TAO2 C-terminal fragments, and other recombinant proteins were expressed and purified from bacteria essentially as described previously (20). Induction of expression was with 30–300  $\mu$ M isopropyl-1-thio- $\beta$ -D-galactopyranoside at 25 or 30 °C for 4–16 h, based on individual optimizations.

**Immunoprecipitation and Affinity Purification from Transfected 293 Cells and Sf9 Cells**—pCMV5-Myc-TAO2 constructs were transfected into 293 cells using calcium phosphate (21). After 48 h, cells were lysed (22), and transfected proteins were detected by anti-Myc Western blotting. Lysate volumes containing equal amounts of expressed protein were used for subsequent immunoprecipitation with anti-Myc antibodies for kinase assays. Sf9 lysates containing His<sub>6</sub>-TAO2 proteins were incubated with Ni<sup>2+</sup>-nitrilotriacetic acid-agarose in buffer containing 0.15 M NaCl and 0.5 mM DTT and washed with 0.3 M NaCl, 0.5 mM DTT, and 10 mM imidazole. Bound proteins were eluted with 250 mM imidazole in buffer and subjected to Western blotting with an anti-MEK3 antibody (23).

**In Vitro Kinase Assays**—Kinase assays contained 50 mM HEPES, pH 8, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 0.5 mg/ml myelin basic protein (MBP), and 100  $\mu$ M ATP ( $\gamma$ -<sup>32</sup>P]ATP, 2–7 cpm/fmol). Reactions were halted with 10  $\mu$ l of 5 $\times$  electrophoresis sample buffer, followed by boiling, and 20  $\mu$ l were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. For linked kinase assays, 50–250 ng of recombinant TAO2 protein was incubated with 50 ng of MEK proteins in 30  $\mu$ l for 60 min at 30 °C; 5  $\mu$ l of the reactions were added to second reactions containing K52R ERK2, p38, or GST-SAPK $\beta$  (23, 24) at 10  $\mu$ g/ml. Phosphoamino acids were determined as described (25).

**In Vitro Binding Assays**—For binding assays involving GST-tagged TAO2 fragments and His<sub>6</sub>-tagged MEK proteins, 3  $\mu$ g of each GST fusion protein or GST alone was incubated with glutathione-agarose beads at 4 °C in the presence of 0.1 mg/ml bovine serum albumin for 30 min and washed with 0.1 M NaCl in 50 mM Tris, pH 7.4. 5  $\mu$ g of His<sub>6</sub>-tagged protein were incubated with the beads in the presence of 0.1 mg/ml bovine serum albumin and 0.1 M NaCl at 4 °C for 1 h. The beads were washed with 0.3 M NaCl, 0.1% Triton X-100, and 50 mM Tris, pH 7.4. Bound proteins were released with 1 $\times$  SDS electrophoresis sample buffer and subjected to anti-His<sub>6</sub> Western blotting. Similar

binding assays were performed for His<sub>6</sub>-TAO2-(314–451) and GST-tagged MEK proteins.

## RESULTS

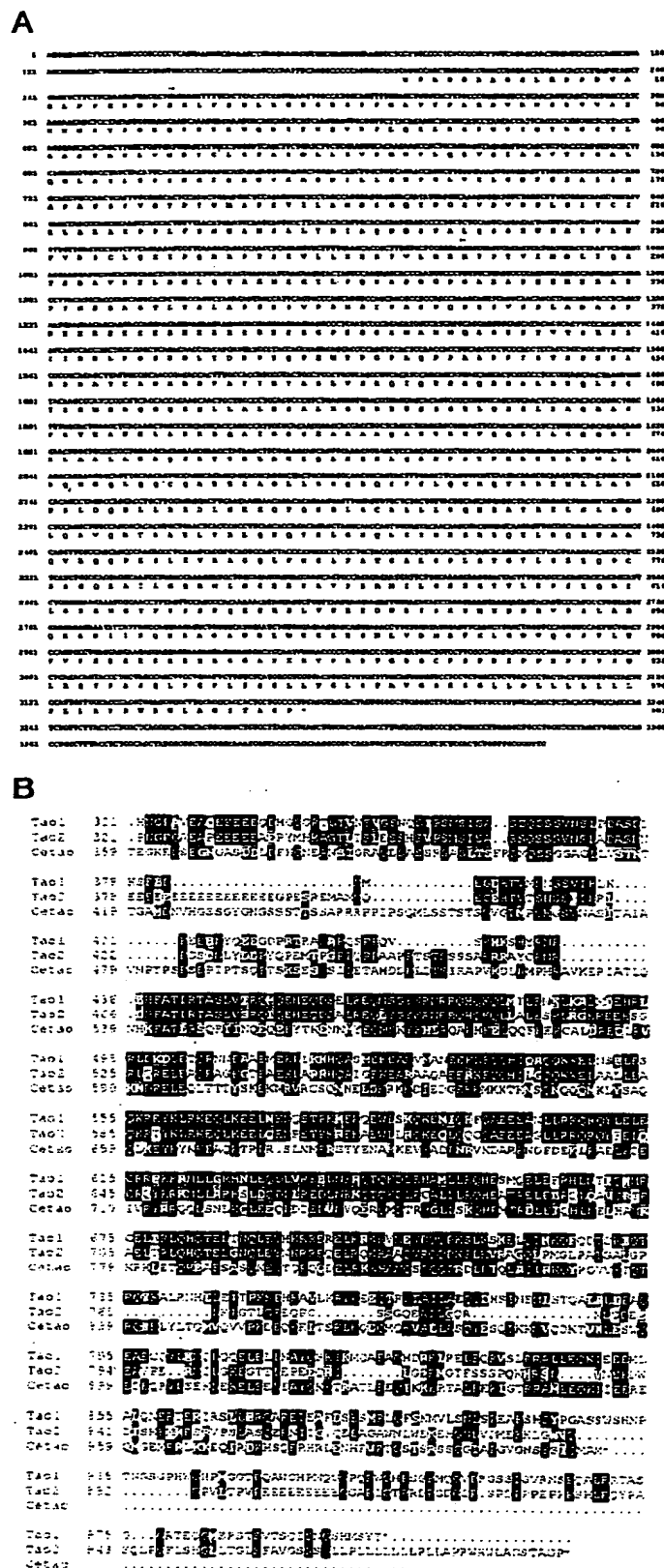
**Isolation of TAO2 cDNAs**—Degenerate oligonucleotide primers designed from the sequence of the *Saccharomyces cerevisiae* Ste20p kinase were used in PCR to amplify fragments of related protein kinases from rat cDNAs. One PCR product was used in isolating overlapping cDNAs from two rat brain cDNA libraries that encoded two protein kinases, TAO1 (8) and the related kinase TAO2, described here. The assembled TAO2 cDNA predicted an open reading frame of 993 amino acids (Fig. 1A). The presumed start codon is located at base 193 and is preceded by an in-frame stop codon at base 145. The longest 3'-untranslated region was 1317 base pairs in length, including a poly(A) track at its end, ~1.3 kilobase pairs 3' to the stop codon (not shown).

**Amino Acid Sequence of TAO2**—The deduced TAO2 protein has a calculated molecular mass of 114 kDa. The serine/threonine protein kinase catalytic domain is at its N terminus. In its 690 C-terminal residues, TAO2 contains a possible nucleotide binding site, a serine-rich region, and a proline and leucine-rich region, all shared with TAO1, and an unbroken stretch of 17 glutamic acid residues unique to TAO2. Like TAO1, TAO2 does not appear to contain a small G protein binding consensus motif found in several other Ste20p relatives (14). The TAO2 protein kinase domain displays 90 and 63% identity to TAO1 and the *Caenorhabditis elegans* TAO ortholog (CeTAO, accession number U32275), respectively (not shown). TAO2 displays marked similarities to TAO1 and the *C. elegans* kinase outside the catalytic domain (Fig. 1B).

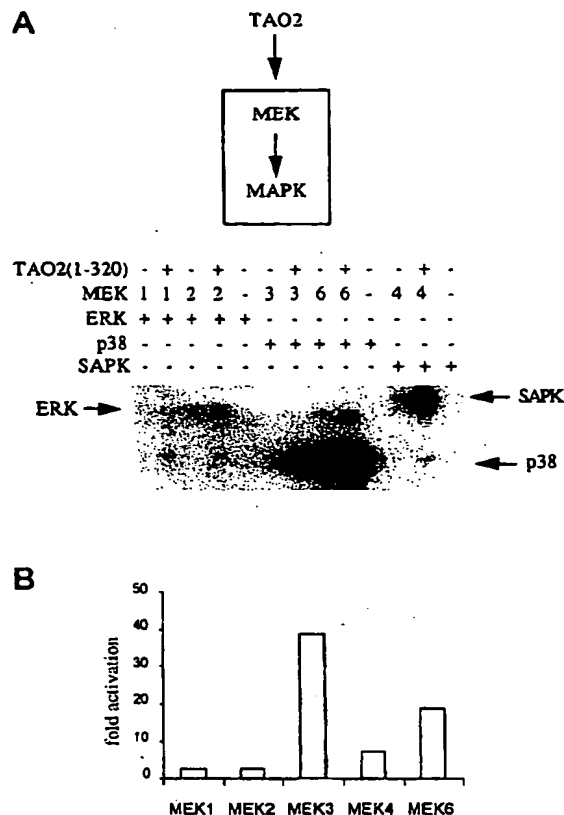
**Expression and Activity of TAO2**—Truncated, recombinant TAO2-(1-320) purified from Sf9 cells phosphorylated MBP with a specific activity of  $0.6 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ . The full-length protein purified on MonoQ had lower intrinsic activity, about 10% of the truncated enzyme (not shown). Kinase-deficient mutants, His<sub>6</sub>-TAO2D169A expressed in Sf9 cells and purified on MonoQ or His<sub>6</sub>-TAO2K57A expressed in bacteria, were inactive toward MBP *in vitro*. TAO2 and TAO2-(1-320) expressed in either Sf9 or mammalian cells autophosphorylated extensively on serine and threonine residues (data not shown).

**TAO2 Activates MEK3, MEK4, and MEK6 *In Vitro***—TAO1 was previously shown to activate MEKs 3, 4, and 6 *in vitro*. We therefore examined the ability of TAO2 to activate MEK family members. TAO2-(1-320) produced in Sf9 cells was subjected to a linked kinase assay by incubating it with recombinant MEKs produced in bacteria in the presence of ATP. Aliquots of the first stage reactions were transferred to second reactions to measure the phosphorylation of appropriate MAP kinase substrates by the recombinant MEKs (Fig. 2A). TAO2-(1-320) activated MEK3 and MEK6 40- and 20-fold, respectively, toward their substrate p38 (Fig. 2B). TAO2 also increased the ability of MEK4 to phosphorylate its substrate SAPK by 7-fold. TAO2-(1-320) was unable to increase the activity of MEK1 or MEK2 toward their substrate K52R ERK2. Full-length TAO2 displayed about 20% of the MEK3-activating ability of TAO2-(1-320), consistent with its lower activity toward MBP. Neither TAO2 mutants D169A nor K57A activated any of the MEKs (data not shown). TAO2-(1-320) expressed in 293 cells also enhanced the ability of MEK3 and MEK4 to phosphorylate their substrates (not shown).

**TAO2 Interacts with MEK3**—We found that recombinant TAO1 copurified with MEK3 endogenous to Sf9 cells, and overexpressed TAO1 interacted with MEK3 in 293 cells (8). These observations led us to investigate whether TAO2 has similar properties. TAO2 proteins overexpressed in Sf9 cells (Fig. 3A) were purified on nickel resin and immunoblotted for MEK3. As



**FIG. 1. Nucleotide and protein sequence of TAO2. A**, the complete amino acid sequence of TAO2 is indicated below the nucleotide sequence. Most of the 3'-untranslated region is not shown but was deposited in GenBank™ (accession number AF140556). The boundaries of the minimal catalytic domain are denoted by the arrows above residues 25 and 285. **B**, the alignment of the noncatalytic domains of TAO1 and CeTAO, the *C. elegans* TAO ortholog (8), with TAO2 residues



**FIG. 2. TAO2 has MEKK activity. A**, linked kinase assays were used to measure activation of various MEK family members by recombinant TAO2(1-320) purified from Sf9 cells. Phosphorylation of appropriate MAP kinase substrates by the MEK family members in second reactions are shown. **B**, data represented in **A** have been quantitated and are plotted as fold activation of MEKs by TAO2(1-320). One of five similar experiments is shown.

a control, Sf9 cell lysates not expressing TAO2 were processed similarly. MEK3 endogenous to Sf9 cells was associated with full-length, wild-type TAO2 (Fig. 3B, lane 2; Fig. 3C, lane 1) but not TAO2D169A (Fig. 3C, lane 2), TAO2(1-320) (Fig. 3B, lane 3), or beads incubated with lysates from uninfected Sf9 cells (Fig. 3B, lane 1; Fig. 3C, lane 3). These results demonstrated that TAO2 binds to MEK3, the interaction is mediated by the noncatalytic region of the protein, and TAO2 catalytic activity enhances MEK3 binding to the full-length protein.

To determine the domain in TAO2 that mediates the interaction with MEK3, the series of fragments that span the noncatalytic domains of TAO2 were expressed as GST fusion proteins and tested for their abilities to bind His<sub>6</sub>-MEK3 *in vitro* (Fig. 3D). The MEK3 binding domain was localized to an ~135-residue region, residues 314–451, just C-terminal to the TAO2 catalytic domain. This region was further subdivided, but all of the shorter fragments containing residues 395–451 were degraded. TAO2(314–377), which precedes the polyglutamic acid region, was insufficient for MEK3 binding.

**TAO2 Binds MEKs 3 and 6 in Vitro but Not MEKs 1, 2, or 4**—To investigate the binding specificity of the TAO2 MEK binding domain, His<sub>6</sub>-tagged MEK proteins were compared for their capacity to bind to TAO2(314–451). The TAO2 fragment bound MEK6 in addition to MEK3 but not MEK1, MEK2, or MEK4 (Fig. 4, A and D). Binding to both MEK3 and MEK6 is

321–993 demonstrates significant similarity outside their kinase domains. Identical residues are boxed in black and conserved residues are shaded.

## MEK Binding Domain of TAO2

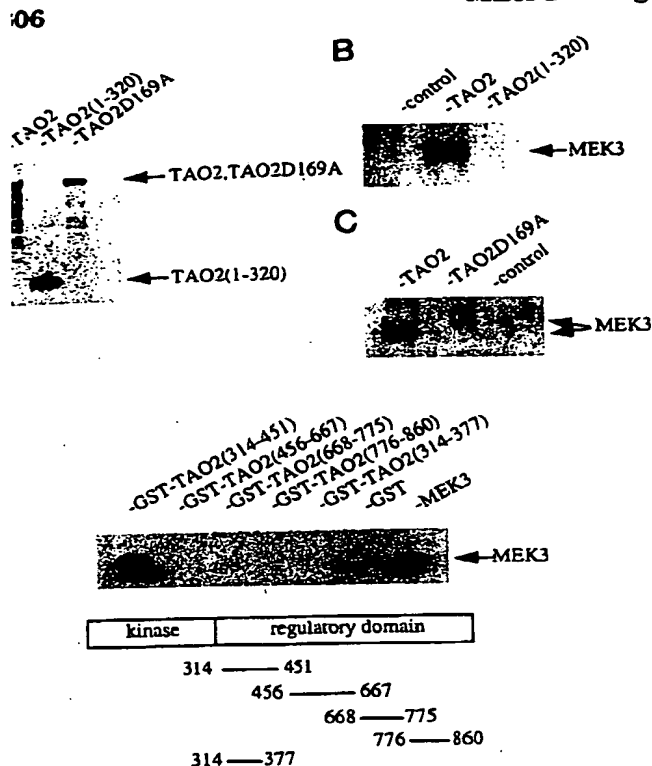


Fig. 3. Identification of the TAO2 MEK binding domain. A, His<sub>6</sub>-tagged TAO2, TAO2-(1-320), and TAO2D169A were expressed in separate batches of Sf9 cells, and the proteins were detected with an antibody that recognizes the N-terminal epitope. Comparable amounts of TAO2 proteins were detected in each lysate. B and C, His<sub>6</sub>-tagged TAO2, TAO2-(1-320), and TAO2D169A were purified from cell lysates and subjected to Ni<sup>2+</sup>-nitrilotriacetic acid-agarose and subjected to anti-MEK3 Western blotting to detect associated MEK3 that was endogenous to Sf9 cells. Lysates from Sf9 cells not expressing recombinant protein were used as a control. One of three comparable experiments is shown. The same experiment was also performed in Sf900 cells with a similar result. D, TAO2 C-terminal fragments were expressed as GST fusion proteins in bacteria and tested for MEK3 binding activity. MEK3 binding was measured by immunoblotting the proteins bound to the beads with anti-His<sub>6</sub> antibodies. His<sub>6</sub>-MEK3 was loaded in the last lane as a positive control. Binding reactions were performed from five to eight lanes for the various TAO2 fragments.

consistent with their significant sequence similarity compared with the other MEK family members. Chimeric proteins generated from MEK6 and MEK1 (Fig. 4B) were used to determine the portion of the MEK that binds to the TAO2 domain. His<sub>6</sub>-MEK1/6 was unable to bind to TAO2-(314-451), whereas GST-MEK6/1 is as efficient as GST-MEK6 in binding to the TAO2 fragment (Fig. 4, C and D).

As noted earlier, catalytically defective TAO2 was deficient in MEK3 binding. To explore the underlying reason, we asked whether autophosphorylation of TAO2 might have an effect on its ability to bind to MEK3. The MEK3 binding fragment of TAO2 was autophosphorylated by the catalytic domain of TAO2 on both serine and threonine residues (Fig. 5, A and B). Thus, first phosphorylated TAO2-(314-451) with TAO2-(1-320) for different lengths of time to determine whether phosphorylation would alter its binding activity. Different concentrations of ATP and Mg<sup>2+</sup> were also tested in the binding assay. The effect of the autophosphorylation state or [ATP-Mg<sup>2+</sup>] on MEK3 binding activity was observed (Fig. 5C). To determine whether MEK3 kinase activity was necessary for binding to TAO2, the binding of kinase-inactive MEK3 (K64M) was tested (Fig. 5D). This defective mutant binds to TAO2 as well as wild-type MEK3, suggesting that MEK3 kinase activity is dispensable for interaction with TAO2.

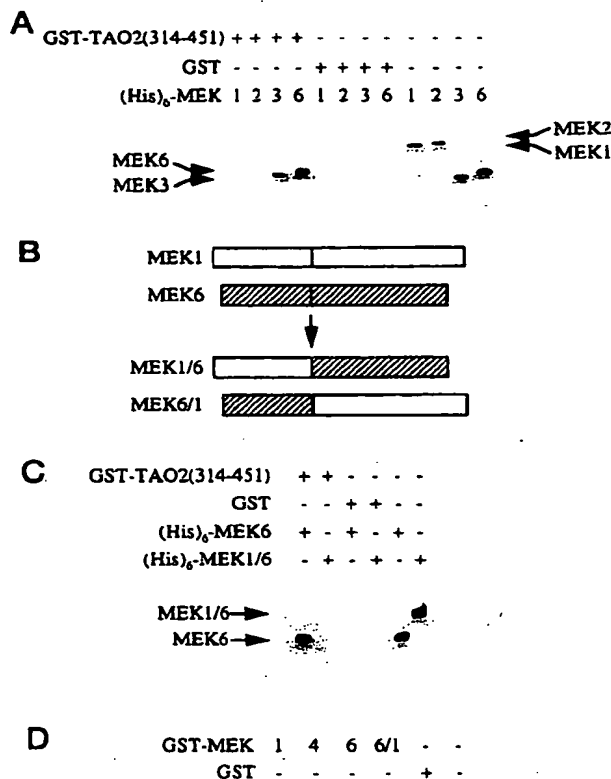


Fig. 4. Specificity of MEK binding to TAO2-(314-451). A, binding of GST-TAO2-(314-451) to His<sub>6</sub>-tagged MEK family members. The MEK family members associated with bead-bound GST-TAO2-(314-451) were detected using anti-His<sub>6</sub> antibodies. The last four lanes contain purified MEK1, -2, -3, and -6, respectively, to show their positions on the gel. One of three similar experiments is shown. B, chimeric proteins were derived from MEK6 and MEK1. MEK1/6 consists of the N terminus of MEK1 and the C terminus of MEK6, whereas the reciprocal chimera MEK6/1 consists of the N terminus of MEK6 and the C terminus of MEK1. MEK1/6 chimeras were used to determine the portion of MEK6 involved in TAO2 binding as described in the legend to A. One of two similar experiments is shown. C, binding of His<sub>6</sub>-TAO2-(314-451) to His<sub>6</sub>-tagged MEK6 or MEK1/6. MEK1/6 chimeras were used to determine the portion of MEK6 involved in TAO2 binding as described in the legend to A. One of two similar experiments is shown. D, binding of His<sub>6</sub>-TAO2-(314-451) to GST-tagged MEK1, -4, -6, or -6/1. Binding to MEK4 was tested, and the tags on the MEK family members and TAO2 proteins were reversed to confirm that the tags had no effect on their protein-protein association. Binding was detected as in A. His<sub>6</sub>-TAO2-(314-451) was loaded in the last lane as a positive control. One of two similar experiments is shown.

## DISCUSSION

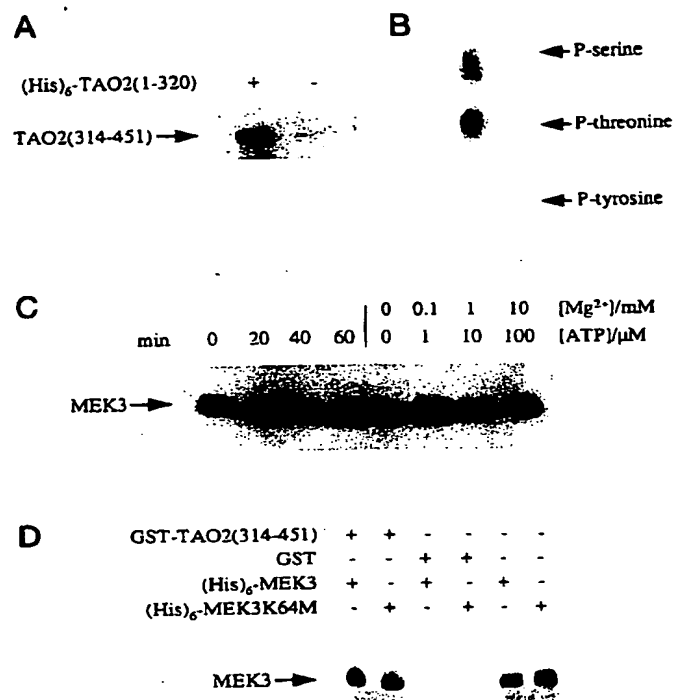
We isolated cDNAs encoding TAO2, a homolog of the previously reported TAO1 (8). We found that TAO2, like TAO1, activated MEKs in the stress-responsive MAP kinase pathways and displayed stable binding to MEK3 endogenous to Sf9 cells. In examining TAO2 expressed in Sf9 cells, we found that the full-length enzyme was significantly less active than the truncated kinase. Thus, the full-length protein was inhibited relative to its truncated forms. Subsequent work indicated that full-length TAO1 is also less active than proteins with C-terminal domain truncations. The inherently higher activity of fragments of TAO1 and -2 suggested that we may have removed an autoinhibitory or pseudosubstrate domain. However, we have not yet identified such a domain, as none of the recombinant fragments from the putative regulatory domain of TAO2 inhibited the activity of its catalytic domain (not shown).

Because TAO2 was purified in a stable complex with MEK3



## MEK Binding Domain of TAO2

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**FIG. 5.** Neither MEK activity nor autophosphorylation of its MEK binding domain by TAO2 is required for MEK binding to TAO2. **A**, 5 μg of TAO2(314–451) was incubated with or without 1 μg of His<sub>6</sub>-TAO2(1–320) under phosphorylating conditions. The reaction mixture was resolved by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography to detect phosphorylation of the MEK binding site in TAO2. One of three similar experiments is shown. **B**, the labeled TAO2(314–451) band in **A** was excised and subjected to phosphoamino acid analysis. Migration of phosphoamino acids was determined by ninhydrin staining of unlabeled standards. **C**, GST-TAO2(314–451) was incubated with His<sub>6</sub>-TAO2(1–320) in the presence of Ni<sup>2+</sup>-nitrilotriacetic acid-agarose under phosphorylating conditions for different lengths of time. Reactions were stopped by transferring to 4 °C and sedimenting the beads to remove the active TAO2 fragment. Supernatants were subjected to binding assays with His<sub>6</sub>-MEK3 (*left*) as described in the legend to Fig. 4A. The indicated concentrations of ATP and Mg<sup>2+</sup> were tested for their effects on binding (*right*). One of two similar experiments is shown. **D**, binding of GST-TAO2(314–451) to His<sub>6</sub>-tagged MEK3 or kinase-defective MEK3 (K64M). Binding was detected as described in the legend to Fig. 4A. One of three similar experiments is shown.

endogenous to Sf9 cells, we localized the MEK binding domain to a small, ~135-amino acid fragment, residues 314–451, just C-terminal to the catalytic domain of TAO2. The N-terminal half of this fragment, residues 314–377, did not bind to MEK3. Because TAO1 and TAO2 both bind MEKs but TAO1 has no polyglutamate stretch, it seems unlikely that these residues participate in MEK binding. Thus, residues from 395 to 451 are most likely required for the stable association with MEKs. These results are consistent with the weak binding of TAO1(1–416) to MEK3 compared with the strong binding displayed by full-length TAO1 (8) and suggest that residues 404–446, which are well conserved between TAO2 and TAO1, contain the MEK binding domain.

Because TAO1 and -2 can activate MEKs 3, 4, and 6 *in vitro*, we determined the specificity of the MEK binding domain of TAO2. We found that TAO2 binds to MEK3 and MEK6, but not to MEK4, despite the fact that MEK4 is an *in vitro* substrate. The N terminus of the MEK is required for this binding, whereas the C terminus is dispensable. This behavior may be a general property of the organization of MAP kinase cascades. The N termini of other MEK family members contain binding

domains for proteins in their cascades. MEK1 binds with high affinity to ERK2 through a basic motif N-terminal to its catalytic domain. MEK1 has been proposed to retain ERK2 in the cytoplasm of unstimulated cells through binding to this site (26), and activation of ERK2 may be impaired if this binding domain is absent.<sup>2</sup> MEK4 is reported to require its N-terminal extension to interact with both MEKK1, an activator, and its substrates, JNK/SAPKs (17). An inhibitory interaction between MEK4 and JNK/SAPKs has also been mapped to this N-terminal domain.<sup>3</sup> This suggests that the stable association of MEK3 or MEK6 with TAO proteins will link their physiological functions to p38 but not JNK/SAPK pathways by restricting their intracellular targets. Future biochemical studies will focus on determining the functions of the other domains of TAO1 and TAO2.

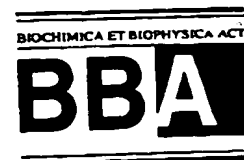
**Acknowledgments**—We thank Lori Christerson and Alf Dang (UT Southwestern) for critical reading of the manuscript, Lori Christerson and Colleen Vanderbilt for providing MEK1/6 and 6/1 chimeras, Signal Pharmaceuticals for the MEK6 cDNA, Alf Dang for help with data analysis, and Peiqun Wu and Don Arnette for MEK proteins. We particularly thank Jim Boulter (UCLA) for providing several rat cDNA libraries.

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## Characterization of $pI_{Cl_n}$ phosphorylation state and a $pI_{Cl_n}$ -associated protein kinase

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### Abstract

$pI_{Cl_n}$  is a ubiquitous cellular protein that has been proposed to be a volume-sensitive  $Cl^-$  channel or a channel regulator. Detailed biochemical, cellular and molecular characterization of  $pI_{Cl_n}$  is required to understand its function. Our goal in the present investigation was to define further the biochemical properties of  $pI_{Cl_n}$  and the proteins that associate with it. Immunoprecipitation of  $pI_{Cl_n}$  from  $^{32}P$ -orthophosphoric acid-labeled C6 glioma cells revealed that the protein is phosphorylated constitutively, primarily on serine residues. Protein kinase activity was detected in  $pI_{Cl_n}$  immunoprecipitates, revealing that a constitutively active protein kinase co-precipitates with  $pI_{Cl_n}$ . A specific association between  $pI_{Cl_n}$  and a protein kinase was also observed in affinity assays using a recombinant GST- $pI_{Cl_n}$  fusion protein. The  $pI_{Cl_n}$ -associated kinase displayed broad substrate specificity and was inhibited in a concentration-dependent manner by heparin, zinc and 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzene (DRB). These characteristics resembled those of casein kinase I and II. The  $pI_{Cl_n}$ -associated kinase was not recognized, however, by antibodies against these two enzymes. Association of the kinase with  $pI_{Cl_n}$  was disrupted by increasing concentrations of NaCl in the washing buffer, suggesting that electrostatic interactions are involved in kinase binding. Mutagenesis experiments corroborated this observation. Truncation of  $pI_{Cl_n}$  demonstrated that two highly charged clusters of acidic amino acid residues are both necessary and sufficient for kinase binding. Phosphopeptide mapping demonstrated that  $pI_{Cl_n}$  contains at least two phosphorylated serine residues that are located on trypsin cleavage fragments rich in acidic amino acid residues. We propose that the kinase or a kinase binding protein binds to acidic amino acids located between D101 and Y156 and phosphorylates nearby serine residues. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Anion channel; Cell volume regulation; Cell swelling

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### 1. Introduction

$I_{Cl_n}$  is a  $Cl^-$  current induced by overexpression of  $I_{Cl_n}$  cRNA in *Xenopus* oocytes [1]. The cDNA re-



sponsible for inducing  $I_{Cln}$  was cloned originally from MDCK cells, but the gene is found ubiquitously in mammalian cells [1–4], and is also present in evolutionarily more ancient vertebrates such as *Xenopus laevis* [2] and zebra fish [5]. When expressed in oocytes, the channel responsible for  $I_{Cln}$  is constitutively active, has broad anion selectivity, is outwardly rectifying, is inactivated by strong depolarization, and is blocked by extracellular nucleotides such as cAMP and ATP [1]. These characteristics resemble those of an ubiquitous, swelling-activated, outwardly rectifying anion current,  $I_{Cl, swell}$ . We have termed the channel responsible for  $I_{Cl, swell}$  VSOAC (Volume-Sensitive organic Osmolyte/Anion Channel). VSOAC appears to mediate swelling-induced efflux of both  $Cl^-$  and uncharged organic osmolytes [6,7]. The channel giving rise to  $I_{Cln}$  has been proposed to be the channel responsible for  $I_{Cl, swell}$  (i.e., VSOAC) [2,8,9]. More recently, however, it has been suggested that VSOAC and the  $I_{Cln}$  channel are distinct molecular species [10,11].

The  $I_{Cln}$  protein,  $pI_{Cln}$ , is an abundant and ubiquitous cellular protein (e.g., [1–4]). In their original study, Paulmichl et al. [1] argued that  $pI_{Cln}$  forms a  $Cl^-$  channel with a  $\beta$  barrel pore structure. Subsequently, however, Krapivinsky et al. [2] concluded that  $pI_{Cln}$  is not a channel, but is instead a channel regulator. Their conclusion was based on the observation that  $pI_{Cln}$  is an acidic, water soluble protein located primarily in the cell cytoplasm. Krapivinsky et al. [2] suggested that overexpression of  $pI_{Cln}$  in oocytes activated an endogenous VSOAC [2,12,13].

An understanding of the role, if any, of  $pI_{Cln}$  in VSOAC activity and volume homeostasis requires extensive biochemical, cellular and molecular characterization of the protein. Our goal in the present investigation was to define further the biochemical properties of  $pI_{Cln}$  and the proteins that associate with it.  $pI_{Cln}$  contains consensus sites for phosphorylation by a number of different kinases (see Section 4 and Fig. 7). This suggested that  $pI_{Cln}$  is a phosphoprotein and that phosphorylation may control its functional properties. We demonstrate here that  $pI_{Cln}$  is phosphorylated in vivo, that it associates selectively in vitro with a constitutively active serine–threonine kinase, and that clusters of acidic amino acids on the protein are important for kinase binding.

## 2. Experimental

### 2.1. Cell culture

Rat C6 glioma cells were cultured to near confluency in Eagle's minimal essential medium (MEM; Gibco, Gaithersburg, MD) with 10% fetal bovine serum (FBS; HYCLONE, Logan, UT) and penicillin/streptomycin as described previously [14].

### 2.2. Antibody production

A fusion protein consisting of full length  $pI_{Cln}$  cloned from rat C6 glioma cells fused to glutathione S-transferase (GST) was generated in BL21 *E. coli* using a commercially available kit (Pharmacia Biotech, Piscataway, NJ). The GST- $pI_{Cln}$  fusion protein was purified using Glutathione Sepharose 4B,  $pI_{Cln}$  was cleaved from GST with thrombin, and the protein was injected intradermally and intramuscularly into rabbits to generate polyclonal antibodies. Preimmune and immune sera were harvested and stored frozen at  $-20^\circ\text{C}$ .

### 2.3. Immunoprecipitation

C6 cells were grown to near confluency in 100 mm diameter tissue culture plates, washed briefly with phosphate-buffered saline (PBS; 2.7 mM KCl, 144 mM NaCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 8.1 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4) and scraped into 0.5 ml of ice-cold lysis buffer (20 mM KCl, 50 mM Tris, pH 7.4, 3 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM DTT, 0.5% NP-40, 1 mM  $\text{Na}_3\text{VO}_4$ , 20 mM NaF, pH 7.4) containing protease inhibitors (10  $\mu\text{g}/\text{ml}$  leupeptin, 10  $\mu\text{g}/\text{ml}$  pepstatin, 10  $\mu\text{g}/\text{ml}$  aprotinin, and 1 mM phenylmethanesulfonyl fluoride [PMSF]). After incubating for 20 min on ice, the cell lysate was centrifuged at 14,000 rpm for 10 min.  $pI_{Cln}$  and associated proteins were immunoprecipitated by adding 10  $\mu\text{l}$  of anti- $pI_{Cln}$  polyclonal antiserum to each 0.5 ml of supernatant and incubating for 2 h at  $4^\circ\text{C}$ . Protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added as a slurry of 20  $\mu\text{l}$  for every 0.5 ml of supernatant and incubated for 2 h at  $4^\circ\text{C}$  on a Labquake Shaker (Labindustries, Berkeley, CA). The resin was then washed five times with ice-cold lysis buffer without protease inhibitors. Proteins were dis-

ociated from the resin by boiling for 4 min and then resolved by SDS-PAGE.

In some experiments, cell lysates were pre-cleared by incubation with 10  $\mu$ l of rabbit serum and 40  $\mu$ l of protein A-agarose. After incubation for 1 h, the slurries were centrifuged, and the supernatant recovered for immunoprecipitation of pI<sub>Cln</sub> and associated proteins.

#### 2.4. pI<sub>Cln</sub> affinity assay

GST-pI<sub>Cln</sub> fusion protein was generated as described above. The protein was purified by immobilization on Glutathione Sepharose 4B followed by washing with 1% Triton X-100 in PBS three times, then three times in PBS. Proteins that bind to pI<sub>Cln</sub> were isolated by incubating C6 glioma cell lysates with immobilized GST-pI<sub>Cln</sub> for 30 min. Unbound proteins were removed by washing five times with lysis buffer. Bound proteins were dissociated by boiling for 4 min, and then resolved by SDS-PAGE.

#### 2.5. <sup>32</sup>P labeling of pI<sub>Cln</sub>

C6 glioma cells were grown to near confluency in 100 mm diameter tissue culture plates and washed with phosphate-free MEM. Cells were depleted of phosphate by incubating them in 5 ml of phosphate-free MEM for 3 h at 37°C. The cells were then labeled with <sup>32</sup>P by incubating them for 4 h with 200  $\mu$ Ci/ml of <sup>32</sup>P-orthophosphoric acid.

As discussed below, protein kinase activity was present in pI<sub>Cln</sub> affinity isolates and anti-pI<sub>Cln</sub> immunoprecipitates. <sup>32</sup>P labeling of immunoprecipitated pI<sub>Cln</sub>, immobilized GST-pI<sub>Cln</sub> or other substrate proteins was carried out by incubating 10  $\mu$ l of Sepharose resin, affinity isolates or immunoprecipitates with 25  $\mu$ l of kinase buffer (50 mM KCl, 10 mM Tris, 10 mM MgCl<sub>2</sub>, pH 7.4) containing 5  $\mu$ Ci of <sup>32</sup>P- $\gamma$ -ATP (6000 Ci/mmol) and 20  $\mu$ M ATP. Unless stated otherwise, in vitro <sup>32</sup>P labeling was performed at 30°C for 15 min.

#### 2.6. Phosphopeptide mapping and phosphoamino acid analysis

<sup>32</sup>P-labeled pI<sub>Cln</sub> was resolved by SDS-PAGE, transferred to a nitrocellulose membrane using a

semidry transfer apparatus, and localized by autoradiography. The section of the membrane containing pI<sub>Cln</sub> was cut out and digested with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, St. Louis, MO). Peptides were separated by electrophoresis in the first dimension with an ammonium bicarbonate-based buffer, pH 8.9, and by ascending chromatography in the second dimension using an isobutyric acid buffer [15]. Phosphoamino acid analysis was carried out by digesting the tryptic fragments further with 6 M HCl for 1 h at 110°C. Phosphorylated amino acids were separated by two-dimensional electrophoresis and detected by autoradiography [15].

### 3. Results

#### 3.1. pI<sub>Cln</sub> is phosphorylated constitutively

In order to assess the phosphorylation state of pI<sub>Cln</sub> in vivo, the protein was immunoprecipitated from <sup>32</sup>PO<sub>4</sub>-labeled C6 glioma cells using a polyclonal rabbit anti-pI<sub>Cln</sub> antiserum. As shown in Fig. 1A, pI<sub>Cln</sub> is phosphorylated constitutively. Phosphoamino acid analysis revealed that the protein was phosphorylated primarily on serine residues (Fig. 1B). Exposure of C6 cells to hypotonic (150 mOsm) or hypertonic (450 mOsm) solutions for 2–5 min had no apparent effect on total <sup>32</sup>P incorporation into pI<sub>Cln</sub> (data not shown).

#### 3.2. pI<sub>Cln</sub> binds to a constitutively active protein kinase

Certain protein kinases bind tightly to their substrates and can be isolated as kinase-substrate complexes [16–18]. In addition, pI<sub>Cln</sub> has been shown to associate tightly and apparently selectively with several cellular proteins [2,19], including the essential myosin light chain [20]. These observations suggested that pI<sub>Cln</sub> might co-immunoprecipitate with the kinase responsible for phosphorylating it. To test this possibility, C6 cell anti-pI<sub>Cln</sub> immunoprecipitates were incubated with <sup>32</sup>P-ATP in kinase buffer containing phosvitin as a kinase substrate (discussed below). As shown in Fig. 2A, phosvitin was phosphorylated under these conditions. The kinase activity

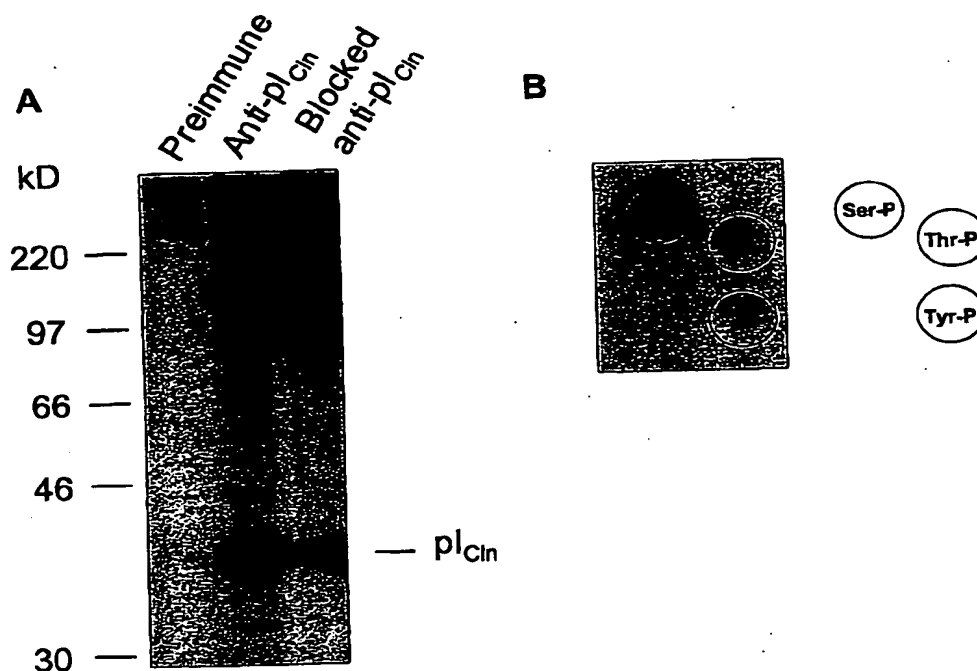


Fig. 1.  $pI_{Cln}$  is a phosphoprotein. (A) C6 glioma cells were loaded with  $^{32}P$ -orthophosphoric acid. Immunoprecipitation of  $pI_{Cln}$  and associated proteins was carried out using anti- $pI_{Cln}$  polyclonal antiserum.  $^{32}P$ -labeled proteins were resolved by SDS-PAGE and visualized by autoradiography.  $pI_{Cln}$  has a predicted molecular mass of  $\sim 26$  kDa [1], but appears as a  $\sim 37$ -kDa protein in SDS-PAGE. This anomalous electrophoretic mobility is most likely due to the large number of acidic amino acid residues present in the protein (see Fig. 7). Little or no  $pI_{Cln}$  is immunoprecipitated using preimmune serum or polyclonal antiserum blocked with recombinant  $pI_{Cln}$  (10  $\mu g/\mu l$  antiserum). These experiments were repeated eight times with similar results. (B) Phosphoamino acid analysis indicates that native  $pI_{Cln}$  is  $^{32}P$ -labeled primarily on serine residues. These experiments were repeated 3 times with similar results. The three circles labeled Ser-P, Thr-P, and Tyr-P correspond to the standard positions of phosphorylated serine, threonine and tyrosine, respectively.

was not observed when C6 cells lysates were treated with anti- $pI_{Cln}$  antiserum blocked with excess recombinant  $pI_{Cln}$  (data not shown) or preimmune serum (Fig. 2A).

Protein kinase activity could also be isolated using a  $pI_{Cln}$  affinity assay consisting of a recombinant GST- $pI_{Cln}$  fusion protein immobilized on Sepharose–glutathione resin. C6 cell lysates were incubated with either Sepharose GST- $pI_{Cln}$  or Sepharose GST to assess the specificity of the kinase binding. After washing,  $^{32}P$ -ATP was added to the resins in kinase buffer and incubated for 15 min. Casein was added to the reaction mixture as a kinase substrate (see below). As shown in Fig. 2B, kinase activity was detected with GST- $pI_{Cln}$ , but not with GST alone. Time course studies revealed that maximum kinase activity was observed between 5 and 15

min after adding cell lysates to the affinity resins (data not shown).

The specificity of the kinase binding was assessed further by performing competition experiments. C6 cell lysates were incubated with Sepharose GST- $pI_{Cln}$  for 30 min. After washing, the resin was treated overnight with excess recombinant  $pI_{Cln}$  or with BSA.  $^{32}P$ -ATP was then added in kinase buffer and incubated for 15 min. As shown in Fig. 2C, GST- $pI_{Cln}$  was phosphorylated only in the resins treated with BSA. The ability of excess recombinant  $pI_{Cln}$  to compete the kinase off of the immobilized fusion protein suggests that the interaction between the two proteins is a selective one.

Association of the kinase with  $pI_{Cln}$  occurs via electrostatic interactions. The interaction between  $pI_{Cln}$  and the kinase was disrupted by washing the

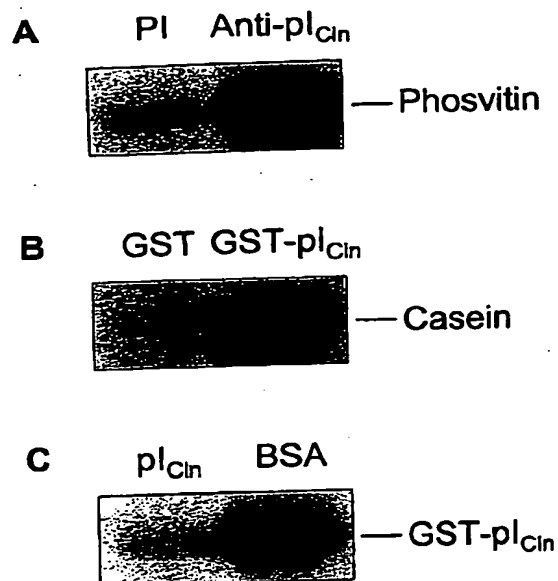


Fig. 2. A constitutively active protein kinase associates with  $pI_{Cln}$ . (A)  $pI_{Cln}$  and associated proteins were immunoprecipitated from pre-cleared C6 glioma cell lysates using anti- $pI_{Cln}$  polyclonal antiserum. Immunoprecipitates were incubated for 15 min with 200  $\mu$ Ci/ml of  $^{32}$ P- $\gamma$ -ATP in kinase buffer containing 0.6 mg/ml phosvitin (used as a kinase substrate; see Fig. 4).  $^{32}$ P-labeled phosvitin was resolved by SDS-PAGE and visualized by autoradiography. Kinase activity is observed when immunoprecipitates are isolated with anti- $pI_{Cln}$  antiserum, but not with preimmune serum. (B) A protein kinase binds selectively to recombinant  $pI_{Cln}$ . C6 glioma cell lysates were incubated for 30 min with immobilized GST- $pI_{Cln}$  fusion protein or immobilized GST. Affinity isolates were incubated with 200  $\mu$ Ci/mol of  $^{32}$ P- $\gamma$ -ATP and 0.4 mg/ml of casein (used as a kinase substrate; see Fig. 4) in kinase buffer for 15 min. Casein phosphorylation was assessed by SDS-PAGE and autoradiography. No kinase activity was isolated from C6 cell lysates incubated with GST alone. Casein is phosphorylated, however, by a protein kinase that binds to GST- $pI_{Cln}$ . These experiments were repeated three times with similar results. (C) The protein kinase can be competed off GST- $pI_{Cln}$  by excess recombinant  $pI_{Cln}$ , but not by bovine serum albumin (BSA). C6 glioma cell lysates were incubated for 30 min with immobilized GST- $pI_{Cln}$  fusion protein. After washing, the affinity isolates were incubated overnight with BSA or recombinant  $pI_{Cln}$  added to a final concentration of 1 mg/ml. The affinity isolates were then washed and incubated for 15 min with 200  $\mu$ Ci/ml of  $^{32}$ P- $\gamma$ -ATP in kinase buffer. Phosphorylation of GST- $pI_{Cln}$  was assessed by SDS-PAGE and autoradiography. These experiments were repeated three times with similar results.

affinity resins with increasing concentrations of NaCl. At concentrations above 200 mM, kinase activity was undetectable (data not shown). This observation sug-

gests an important role for charged amino acid domains in mediating kinase binding.

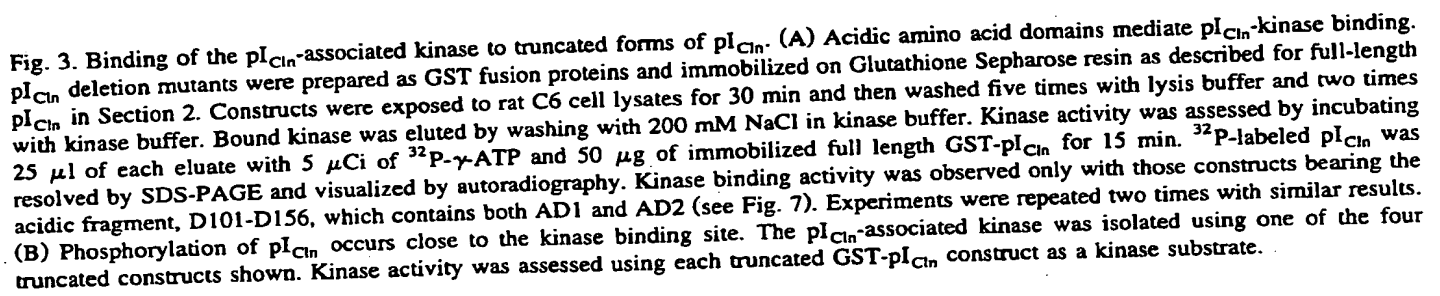
$pI_{Cln}$  contains two clusters of negatively charged amino acids. We refer to these regions as acidic domains 1 and 2 (AD1 and AD2; see Fig. 7). We postulated that these regions might be important for kinase binding, given the fact that kinase binding was sensitive to the salt concentration in the washing buffer. To test this possibility, we generated the following truncated forms of GST- $pI_{Cln}$ : M1-H241 (full length), M1-S100, M1-Y156, D101-Y156, D101-H241, V158-H241. AD1 and AD2 are located between D101 and Y156. The ability of the truncated  $pI_{Cln}$  molecules to bind the kinase was assessed by performing the affinity and kinase assays described above. GST- $pI_{Cln}$  was used as a kinase substrate. As shown in Fig. 3A, no kinase binding was detected on M1-S100 or V158-H241. These two fragments lack AD1 and AD2. Kinase activity was detected, however, on M1-Y156 and D101-H241, which contain both acidic domains. D101-Y156, which is the smallest  $pI_{Cln}$  fragment containing both AD1 and AD2, was also capable of binding the kinase. These results suggest that AD1 and/or AD2 may be important for binding the kinase directly. Alternatively, separate kinase binding protein(s) may interact with AD1 and AD2.

We also assessed the ability of the kinase to phosphorylate various truncated  $pI_{Cln}$  molecules. Affinity and kinase assays were performed as described above. As shown in Fig. 3B, the four truncated constructs that bound the kinase were also phosphorylated by it.

### 3.3. Characteristics of the $pI_{Cln}$ -associated kinase

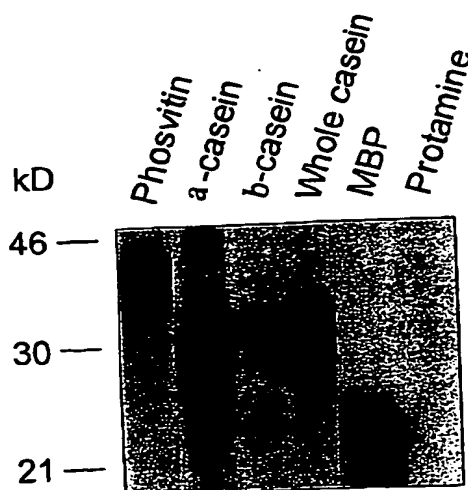
Phosphoamino acid analysis was performed on recombinant  $pI_{Cln}$  phosphorylated by the  $pI_{Cln}$ -associated kinase isolated with either affinity assay or immunoprecipitation. The  $pI_{Cln}$ -associated kinase phosphorylated  $pI_{Cln}$  on serine residues only (data not shown), indicating that it is a serine-threonine protein kinase.

The  $pI_{Cln}$ -associated kinase had a broad substrate specificity and was capable of phosphorylating acidic proteins such as casein,  $\alpha$ -casein and phosvitin, as well as myelin basic protein (MBP) (Fig. 4). Minor phosphorylation of  $\beta$ -casein was also observed. The



Most protein kinases require the presence of  $Mg^{2+}$  for full activity. As shown in Fig. 5A,  $pI_{Cln}$ -associated kinase activity was increased in a concentration

dependent manner by  $Mg^{2+}$ . Kinase activity was reduced greatly when 10 mM  $Mg^{2+}$  was replaced with 10 mM  $Mn^{2+}$  or 10 mM  $Co^{2+}$ . No kinase activity was detectable when 10 mM  $Ca^{2+}$  replaced  $Mg^{2+}$  (Fig. 5A).



4. The  $pI_{Cln}$ -associated kinase has broad substrate specificity. Kinase activity was isolated by affinity assay. The kinase was then eluted from the affinity isolates by incubating for 15 min in kinase buffer containing 200 mM NaCl. Substrate specificity was assessed by adding to the supernatant 200  $\mu$ Ci/ml of  $\gamma$ -ATP and 0.4 mg/ml of various substrate proteins. After a 1 min incubation, the proteins were resolved and visualized by SDS-PAGE and autoradiography. This experiment was repeated three times with identical results. A similar substrate specificity was observed when kinase activity was isolated by immunoprecipitation (data not shown).

$pI_{Cln}$  has consensus sequences for phosphorylation by protein kinase A, C and G, and casein kinase I and II (CKI and CKII) [21,22]. Y151 and Y156 may also

be potential sites for phosphorylation by tyrosine kinases [23,24] (see Fig. 7). Of these kinases, only CKI and CKII are in general constitutively active. In addition, CKI and CKII preferentially phosphorylate acidic proteins [25], and they have frequently been isolated by co-immunoprecipitation with their substrates [16–18]. We, therefore, examined the sensitivity of the  $pI_{Cln}$ -associated kinase to heparin, zinc and 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB), which are known inhibitors of CKI and CKII [26–29]. As shown in Fig. 5B, all three drugs inhibit

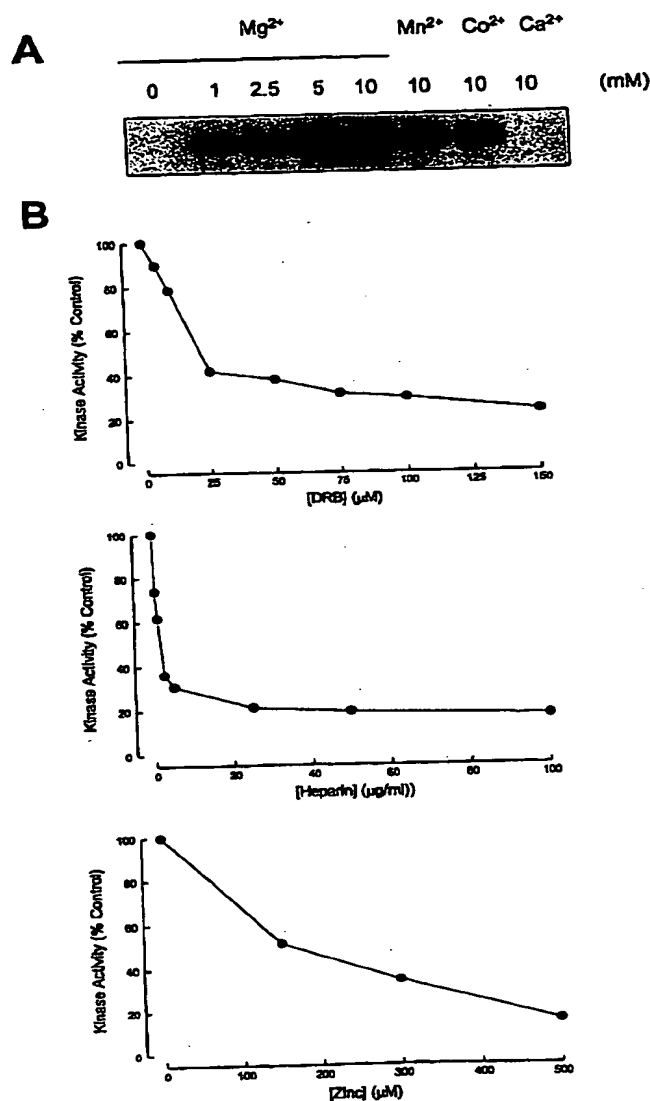


Fig. 5. Pharmacological characteristics of the  $pI_{Cln}$ -associated kinase. (A) Kinase activity requires the presence of  $Mg^{2+}$ .  $pI_{Cln}$ -associated kinase activity was isolated by affinity assay. Affinity isolates were incubated for 15 min in kinase buffer containing 200  $\mu$ Ci/ml of  $^{32}$ P- $\gamma$ -ATP and various concentrations of divalent cations. Phosphorylation of GST- $pI_{Cln}$  was assessed by SDS-PAGE and autoradiography. Divalent cations supported kinase activity with the following potency:  $Mg^{2+} \gg Co^{2+} > Mn^{2+} \gg Ca^{2+}$ . This experiment was repeated two times with similar results. (B) The  $pI_{Cln}$ -associated kinase is inhibited by CKI and CKII inhibitors. Kinase activity was isolated by affinity assay, and affinity isolates were incubated for 15 min in kinase buffer containing 200  $\mu$ Ci/ml of  $^{32}$ P- $\gamma$ -ATP and various concentrations of DRB, heparin or zinc. Proteins were resolved by SDS-PAGE and visualized by Coomassie staining of the gel. The protein band corresponding to GST- $pI_{Cln}$  was cut out of the gel and the radioactivity quantified by Cerenkov counting. Results are expressed as percent of the control. A similar inhibitor sensitivity was observed with kinase activity isolated by immunoprecipitation (data not shown).

ited kinase activity in a concentration-dependent manner. The maximal inhibition observed for each drug was approximately 80%.

The inhibitor sensitivity of the  $pI_{Cln}$ -associated kinase suggested that it may be either CKI or CKII. To address this possibility directly, we performed Western blot analysis of the  $pI_{Cln}$ -associated kinase isolated by affinity assay using anti-CKI or anti-CKII antibodies.

Mammalian cells possess at least four isoforms of CKI [30]. Casein kinase II is composed of three subunits referred to as  $\alpha$ ,  $\alpha'$  and  $\beta$  [25]. To assess whether CKI and CKII are expressed in C6 cells, lysates from  $\sim 2 \times 10^7$  cells (half of a 10 cm diameter culture dish) were prepared in 1 ml of lysis buffer. Twenty-five microliters of this material were then subjected to Western analysis using antibodies to the conserved CKI kinase domain (kindly provided by Dr. David Virshup, University of Utah [31]) and antibodies to the  $\alpha$ ,  $\alpha'$  and  $\beta$  subunits of CKII (kindly provided by Dr. David Litchfield, Manitoba Institute of Cell Biology [32]). Anti-CKI antibodies recognized 2 proteins in C6 cell lysates (data not

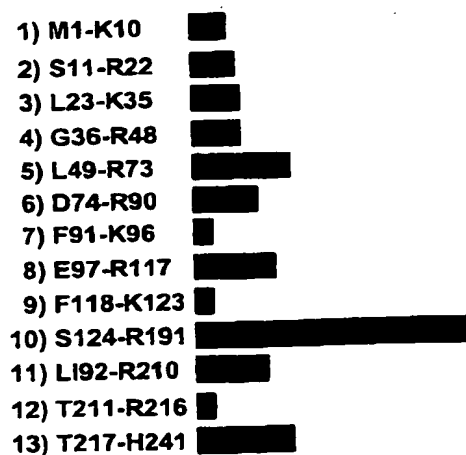
shown). Antibodies to the  $\alpha$ ,  $\alpha'$  and  $\beta$  subunits of CKII each reacted with proteins of the appropriate molecular mass (data not shown).

To determine if CKI or CKII interact with  $pI_{Cln}$ , C6 cell lysates were subjected to GST- $pI_{Cln}$  affinity purification as described previously. The amount of kinase isolated was maximized by preparing lysates from cells grown on ten 10-cm diameter culture dishes ( $\sim 40 \times 10^7$  cells). Affinity-isolated proteins were resolved by SDS-PAGE and subjected to anti-CKI or CKII Western analysis. No proteins were detected (data not shown) even when exposure times were 20-fold longer than those used for Western analysis of total cell lysates. These findings suggest that the CKI or CKII are not the kinases that bind to and phosphorylate  $pI_{Cln}$  in vitro.

### 3.4. Phosphopeptide mapping

Native  $pI_{Cln}$  was phosphorylated by incubating C6 glioma cells with  $^{32}P$ -orthophosphoric acid, and the protein was isolated by immunoprecipitation and subjected to trypsin digestion as described in Section 2.

#### A. Trypsin Cleavage Fragments:



#### B. Phosphopeptide Map:

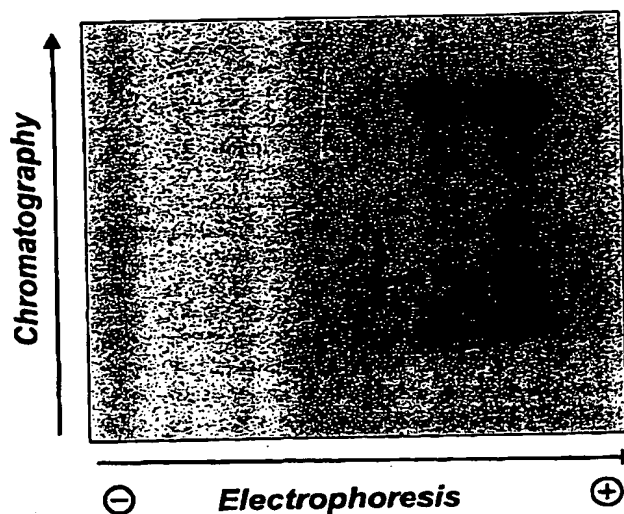


Fig. 6. Phosphopeptide map of native  $pI_{Cln}$ .  $pI_{Cln}$  was immunoprecipitated from  $^{32}P$ -orthophosphoric acid loaded C6 glioma cells. Proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes and digested with trypsin. (A) Predicted trypsin cleavage fragments of rat  $pI_{Cln}$ . Fragments 8 and 10 (solid bars) contain large numbers of acidic amino acid residues, as well as serine residues that are likely sites of phosphorylation. (B) Phosphopeptide map. Trypsin cleavage fragments were separated by electrophoresis and ascending chromatography using thin layer chromatography plates. Phosphopeptides were visualized by autoradiography.

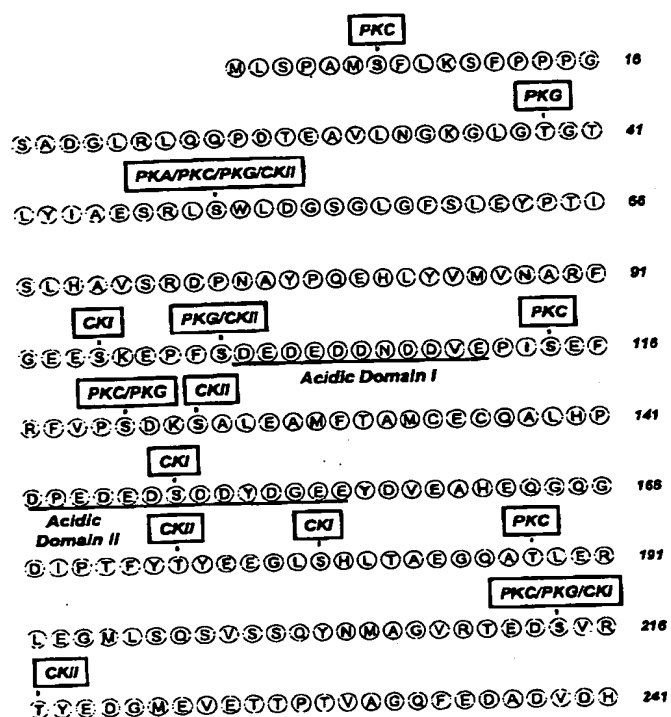


Fig. 7. Amino acid sequence of  $pI_{Cln}$  cloned from rat C6 glioma cells. Consensus sites for phosphorylation by protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), casein kinase I (CKI) and casein kinase II (CKII) were identified using data published by Pearson and Kemp [21] and Kennelley and Krebs [22]. Serine and threonine residues that can putatively be phosphorylated by these kinases are highlighted in bold. The consensus sequences considered were as follows: PKA, R-X-S\* [21]; PKC, S\*/T\*-X<sub>2-6</sub>-R/K<sub>1-3</sub> [22] or K/R-X-S\*/T\* [21]; PKG, R/K-X-S\*/T\* or R/K-X-X-X-S\*T\* [21]; CKI, D/E<sub>2-4</sub>-X<sub>2-6</sub>-S\*/T\* [22]; CKII, S\*/T\*-X-X-E/D-X [21], where X denotes any amino acid and the asterisks denote phosphorylated serine and threonine residues. The amino acid sequence located on the N-terminal side of Y151 and Y156 is highly acidic and enriched in glutamate and aspartate residues. This suggests that these tyrosine residues may be sites of phosphorylation by tyrosine kinases [23,24]. Acidic amino acid domains (AD1 and AD2) are underlined.

In our initial attempts to separate  $pI_{Cln}$  phosphopeptides, we used a formic acid/acetic acid pH 1.9 electrophoresis buffer. For the chromatography phase of the separation, we used a so-called phospho-chromatography buffer [15]. With this buffer system, the phosphopeptides did not separate and remained at the origin. Subsequently, we used a pH 8.9 ammonium bicarbonate electrophoresis buffer and an isobutyric

acid chromatography buffer designed to separate extremely hydrophilic peptides [15]. We obtained good separation of two phosphopeptides with this buffer system (Fig. 6). These results indicate that there are at least two phosphorylated serine residues on  $pI_{Cln}$ . The buffer system required to separate the  $pI_{Cln}$  phosphopeptides indicates that phosphorylated serines are located on acidic trypsin cleavage fragments.

#### 4. Discussion

VSOAC is an outwardly rectifying, swelling-activated anion channel responsible for volume regulatory  $Cl^-$  and organic solute transport [6,7].  $pI_{Cln}$  has been suggested to be VSOAC [8,9] or a VSOAC regulator [2]. More recently, it has been suggested that  $pI_{Cln}$  activates in a non-selective manner a channel that is distinct from VSOAC [10,11]. The controversy raised over  $pI_{Cln}$  is reminiscent of those surrounding the function of min K and phospholemman [33,34]. These controversies have raised fundamental questions about ion channel structure, ion channel regulators and proteins that indirectly modify ion channel function. Resolution of the  $pI_{Cln}$  controversy will require extensive molecular and cellular characterization of this protein using a variety of approaches, including planar lipid bilayer reconstitution. Our goal in the present investigation was to further define the general biochemical properties of  $pI_{Cln}$ , specifically its phosphorylation state.  $pI_{Cln}$  contains a number of consensus sites for phosphorylation by CKI, CKII, protein kinases (PK) A, C, and G and possibly tyrosine kinases (see Fig. 7), suggesting that phosphorylation may control the functional properties of the protein.

As shown in Fig. 1,  $pI_{Cln}$  is constitutively phosphorylated, primarily on serine residues. Immunoprecipitation and affinity assay studies revealed that  $pI_{Cln}$  associates in an apparently selective fashion with a serine/threonine kinase (Fig. 2). This kinase is constitutively active, has broad substrate specificity (Fig. 4), and is inhibited in a concentration-dependent manner by zinc, DRB and heparin (Fig. 5).

The identity of the  $pI_{Cln}$ -associated kinase is unknown. Of all the known kinases for which there are consensus phosphorylation sites on  $pI_{Cln}$ , three can be ruled out as possible candidates: tyrosine kinases,



CKI and CKII (see Fig. 7). PKC or PKG are probably not involved in the *in vitro* phosphorylation of  $pI_{Cln}$ . These kinases require activation by phorbol esters or cGMP, which are not present in our assay buffer. PKA is also unlikely to be important. In general, PKA requires activation by cAMP, but at least one report suggests that it can be constitutively active *in vitro* [35]. We observed, however, that the Walsh peptide (New England Biolabs, Beverly, MA), a highly specific and potent PKA inhibitor [36], had no effect on  $pI_{Cln}$ -associated kinase activity (unpublished observations). Given these findings, it is likely that the  $pI_{Cln}$ -associated kinase is either a new kinase or a kinase for which the consensus phosphorylation sites have not been described and/or cataloged.

Proteins with apparent molecular masses of 17, 29, 72, and 160 kDa bind in what seems to be a selective fashion to  $pI_{Cln}$  [19,20] (see also Ref. [2]). Microsequencing has revealed that p17 is the essential myosin light chain [20]. Krapivinsky et al. [2] have also concluded that actin binds selectively to  $pI_{Cln}$ . The identities of the other  $pI_{Cln}$  binding proteins are presently unknown. It is possible that one of these proteins is the  $pI_{Cln}$ -associated kinase. In gel kinase assays may provide a means to test this idea directly.

We do not know yet whether the kinase isolated *in vitro* is the same one responsible for phosphorylating  $pI_{Cln}$  in intact cells. We attempted to address this issue by performing phosphopeptide mapping on  $pI_{Cln}$  immunoprecipitated from C6 glioma cells, GST- $pI_{Cln}$  or recombinant  $pI_{Cln}$  cleaved from the GST moiety. These three proteins were phosphorylated using the kinase isolated by anti- $pI_{Cln}$  immunoprecipitation. In all cases, separation of the phosphopeptides required a buffer system optimized for acidic proteins. This suggests that the  $pI_{Cln}$ -associated kinase phosphorylates serine residues located on acidic trypsin cleavage fragments similar to that observed for  $pI_{Cln}$  phosphorylated *in vivo* (see Section 3). Each of the three substrate proteins, however, yielded a unique phosphopeptide map (data not shown). These maps were distinct from the map obtained with native  $pI_{Cln}$ . One interpretation of these results is that the kinase observed *in vitro* is distinct from the kinase that phosphorylates  $pI_{Cln}$  in intact cells. It is equally likely, however, that the state of the protein (i.e., bound to antibody, linked to GST or free) and/or the activity of other kinases and phosphatases may influence the

overall phosphorylation state of  $pI_{Cln}$  *in vivo*. Additional studies are required to assess the physiological role played by the  $pI_{Cln}$ -associated kinase in phosphorylating the protein *in vivo*.

Phosphopeptide mapping provided insight into the location of phosphorylated serine residues on  $pI_{Cln}$ . Rat  $pI_{Cln}$  contains 12 predicted trypsin cleavage sites and yields 13 fragments upon trypsin cleavage (Fig. 6). As discussed above, the  $pI_{Cln}$  phosphopeptides we isolated appeared to be highly acidic. Of the 13 trypsin cleavage fragments, three contain large numbers of acidic amino acids: E97-R117 (11 Glu and Asp residues), S124-R191 (20 Glu and Asp residues) and T217-H241 (8 Glu and Asp residues). There are no serine residues on T217-H241. In contrast, there are two serine residues in E97-R117 and three in S124-R191. These findings suggest that S100, S114, S124, S148 and/or S179 are the most likely sites for phosphorylation of rat  $pI_{Cln}$  *in vivo*. Other phosphorylation sites may exist as well. Mutagenesis studies are required to directly determine the sites of phosphorylation.

The putative location of phosphorylated serine residues on acidic peptide fragments is consistent with other observations. As noted in Section 3, association of the kinase with  $pI_{Cln}$  is sensitive to the salt concentration of the washing buffer, suggesting an important role for charged amino acid domains in mediating kinase binding. The  $pI_{Cln}$  truncation experiments shown in Fig. 3A support this idea. The kinase binds only to  $pI_{Cln}$  fragments containing AD1 and AD2. Furthermore, D101-Y156, a 56 amino acid fragment containing both AD1 and AD2, is sufficient for kinase binding. Our results suggest that the kinase binds directly or indirectly via accessory proteins to AD1 and/or AD2 and phosphorylates nearby serine residues. This conclusion is supported by the observation that  $pI_{Cln}$  truncation fragments containing AD1 and AD2 were not only capable of binding the kinase, but were also phosphorylated by it (Fig. 3B).

Clusters of acidic amino acid residues have been shown to be important for several other protein–protein interactions. These include the interaction of eukaryotic transcription factors with components of the transcription machinery [37,38], and the binding of transported proteins to the protein transport system located in the mitochondrial outer membrane [39]. A cluster of acidic amino acid residues on the mam-

nalian endopeptidase furin is thought to be important for internalization of the protein from the plasma membrane and localization to the trans-Golgi network [40].

The physiological role of  $pI_{Cln}$  phosphorylation is unknown. If  $pI_{Cln}$  is the VSOAC channel or a VSOAC regulator, as suggested by Krapivinsky et al. [2] and Gschwentner et al. [8,9], then phosphorylation may control channel activity. In some cell types, phosphorylation and dephosphorylation events have been suggested to play a role in controlling regulatory volume decrease and the activity of volume-sensitive anion channels [41–44]. At least one recent study has suggested that phosphorylation may control swelling-induced activation of VSOAC [45]. In addition, stimulation of PKA or PKC with cAMP and phorbol esters has been shown to enhance VSOAC activity [14,46].

Recent findings of Voets et al. [11,47] and Buyse et al. [10] have challenged the proposed connection between VSOAC and  $pI_{Cln}$ . These investigators observed that  $I_{Cln}$  was induced in oocytes by overexpression of either  $pI_{Cln}$  or the unrelated protein CIC-6. Careful examination of  $I_{Cln}$  demonstrated that its characteristics were distinct from those of the endogenous swelling-activated VSOAC current described in oocytes [10–13]. Furthermore, Buyse et al. [10] and our laboratory (Strange et al., unpublished observations) have observed that oocytes possess an endogenous, constitutively active conductance that has the same characteristics as the current proposed by Paulmichl et al. [1] to arise from expression of a mutant form of  $pI_{Cln}$ . Voets et al. [47] recently attempted to reproduce the mutagenesis studies of Paulmichl et al. [1]. They observed that expression of the so-called AAA mutant induced a current identical to that of wild type  $pI_{Cln}$ . When taken together, these results have led Buyse et al. [10] and Voets et al. [47] to conclude that expression of  $pI_{Cln}$  in oocytes turns on an endogenous anion current that is distinct from VSOAC.

From the above discussion, it is clear that the function of  $pI_{Cln}$  remains obscure. Identification of the  $pI_{Cln}$ -associated kinase, determination of the physiological consequences of  $pI_{Cln}$  phosphorylation, and assessment of the effect of phosphorylation on channel activity induced by  $pI_{Cln}$  in planar lipid bilayers may provide insight into the physiological role of this protein.

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